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Applicability of commercial phage-based products against *Listeria monocytogenes* for improvement of food safety in Spanish dry-cured ham and food contact surfaces

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Title: Applicability of commercial phage-based products against *Listeria monocytogenes* for improvement of food safety in Spanish dry-cured ham and food contact surfaces

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Abstract

The efficacy of bacteriophages as antimicrobials has fostered the approval and commercialization of several products intended to reduce the risk of food contamination by pathogenic bacteria. ListShield™ and Listex™ P100 are bacteriophage preparations that aim to combat *Listeria monocytogenes* in food industries. Here, we assessed the effectiveness of these phage-based products against *L. monocytogenes* strains from food origin. Both, removal of *L. monocytogenes* biofilms and inhibition of growth in Spanish dry-cured ham were evaluated for this bacterium. ListShield™ was effective in lysing 100% of *L. monocytogenes* strains examined, whereas Listex™ P100 lysed only 64% of the same strains. According to the manufacturers' recommendations, ListShield™ was used in a lower concentration than Listex™ P100 for the efficacy studies. Despite these differences, both products were effective in removing 72 h-old biofilms formed on stainless steel surfaces by most of the assayed strains after a 4 h treatment at 12ºC. For some strains, moreover, complete removal of adhered bacteria from 48 h-old biofilms formed on polystyrene surfaces was obtained after 4 h of treatment at 32°C with Listex™ P100. Evaluation of phage-based products as biopreservatives in dry-cured ham showed that the application of Listex™ P100 resulted in a reduction of *L. monocytogenes* contamination below the detection limit (<10 CFU/cm^2) after 24 h at 4°C and 12ºC. Treatment with ListShield™ showed similar results with the exception of samples with a high level of contamination (10^5 CFU/cm^2), in which a reduction of 3.5 log units was achieved after 14 days of incubation at 4ºC. In contrast, the antibacterial activity of ListShield™ decreased in samples stored at 12°C, although a complete elimination of bacteria was observed after 8 days of treatment in low contaminated samples (10^3 CFU/cm^2). All these results suggest that phage-based products can be useful for biocontrol of *L. monocytogenes* in food contact surfaces and dry-cured ham.

Keywords: phage-based products, food safety, *Listeria monocytogenes*, disinfectants, biopreservatives.

1. Introduction

Listeriosis is an important food-borne zoonosis caused by *Listeria monocytogenes*, which mainly affects pregnant women, neonates, babies, immunocompromised patients and the elderly. The severity of this disease (with 20-30% mortality) is caused by the ability of this bacterium to cross human body barriers like blood, brain, intestine and placenta (Vázquez-Boland, et al., 2001). The last report from the European Food Safety Authority (EFSA) informed about a growing trend of listeriosis in the EU over the period 2008-2014. In 2014, there was a 30% increase in the number of outbreaks compared with 2013 and a total of 210 deaths due to this bacterium were reported (EFSA & ECDC, 2016). In the United States, a similar scenario has been described by the Centers for Disease Control and Prevention, with 9 outbreaks and 13 deaths caused by *L. monocytogenes* infections in 2014 (CDC, 2015).

L. monocytogenes is ubiquitous in nature and has been isolated from soil, rivers, plants, and food sources like milk, meat, seafood and vegetables. Additionally, this pathogen is able to survive and form biofilms at refrigeration temperatures, and can often be resistant to disinfectants. Together, these traits might contribute to the persistence of *Listeria* in food-associated environments, with the subsequent risk of food contamination and transmission to humans (Ferreira, Wiedmann, Teixeira, & Stasiewicz, 2014). Usually, the leading vehicle of foodborne listeriosis is ready-to-eat (RTE) foods, which can be contaminated during the post-processing stage (slicing and packaging) and are consumed without further preparation (Vorst, Todd, & Ryser, 2006). *L. monocytogenes* contamination can also be a problem in some traditionally prepared

cured meats, such as dry-cured ham (Levine, Rose, Green, Ransom, & Hill, 2001; Morganti, et al., 2016; Uyttendaele, De Troy, & Debevere, 1999). Spanish dry-cured ham is highly appreciated in many different countries. In 2014, the export of this product reached 36,000 tons, representing an economic value of 300 million euros (OECE, 2016).

Preservation techniques such as high hydrostatic pressure, modified atmospheric packaging, irradiation and biopreservation have been assayed to avoid the development of this pathogen over the product´s shelf life (Hereu, Bover-Cid, Garriga, & Aymerich, 2012; Hoz, Cambero, Cabeza, Herrero, & Ordóñez, 2008; Huq, Vu, Riedl, Bouchard, & Lacroix, 2015; Morales, Calzada, & Núñez, 2006). However, new approaches should be developed to comply with the strict regulatory requirements in some countries. For instance, the US authorities currently have a "zero-tolerance" policy, i.e. no viable cells detected in 25 g for RTE foods. In the EU, there is a zero tolerance for RTE foods that support growth, while *L. monocytogenes* levels should be below 100 CFU/g for products and RTE foods that do not support growth throughout their shelf life (EC, 2013).

Bacteriophages are viruses that infect and kill bacteria. Their potential as antimicrobials against pathogenic bacteria has boosted research concerning their application in many areas of human activity (Sulakvelidze, 2013). Notably, phages have been proposed as disinfectants to remove and prevent biofilm formation in clinical (Donlan, 2009) and food-related environments (Siringan, Connerton, Payne, & Connerton, 2011; Soni & Nannapaneni, 2010). The use of phages as biocontrol agents in food products has the following advantages: i) their abundance in nature including food sources, ii) harmlessness for humans, plants, animals and the environment, iii) no effect on the food organoleptic properties, and iv) no impact on the normal food microbiota (García,

Rodríguez, Rodríguez, & Martínez, 2010). Bacteriophages have been successfully assessed for the control of *L. monocytogenes* in foods such as fruits, RTE products, cheese and milk (Guenther & Loessner, 2011; Leverentz, et al., 2003; Oliveira, et al., 2014; Rodríguez-Rubio et al., 2015). In addition, bacteriophage-derived proteins specific for *Listeria* have recently been used for the detection and control of this pathogen in foods (Schmelcher & Loessner, 2014; Zhang, Bao, Billington, Hudson, & Wang, 2012).

ListShield™ (formely known as LMP-102™) was the first phage-based preparation approved by the FDA and the Environmental Protection Agency, and has also been approved in Canada and Israel. This product is composed of a mixture of six bacteriophages and is intended to be used in food processing plants as a decontaminant of surfaces and equipment as well as for treating foods with a high risk of *L. monocytogenes* contamination. Listex™ P100 is based on bacteriophage P100 (Carlton, Noordman, Biswas, de Meester, & Loessner, 2005) and has been approved as a clean label processing aid in several countries, including USA, Canada, Australia, New Zealand, Switzerland, Israel, Norway and the Netherlands. Listex™ P100 has been shown to reduce *L. monocytogenes* in fish (Soni & Nannapaneni, 2010; Soni, Nannapaneni, & Hagens, 2010), meat (Chibeu, et al., 2013; Iacumin, Manzano, & Comi, 2016), vegetables (Oliveira, Abadias, Colas-Meda, Usall, & Vinas, 2015), fresh fruits and fruit juices (Oliveira, et al., 2014). Of note, these studies point out that the effectiveness of Listex™ P100 is influenced by factors such as phage dose, contact time, storage temperature (Soni & Nannapaneni, 2010) and pH (Oliveira, et al., 2014). Regarding ListShield™, the effectiveness of the phage cocktail has been tested in fresh-cut fruits (Leverentz, et al., 2003) as well as in different RTE products (lettuce, cheese, smoked salmon and frozen foods), leading to a significant reduction of *L.*

monocytogenes contamination without any organoleptic modification (Perera, Abuladze, Li, Woolston, & Sulakvelidze, 2015).

Despite the evidence demonstrating the efficacy of anti-Listeria phage-based commercial products, their potential for improving the safety of Spanish dry-cured ham has not been explored so far. Therefore, the aim of this study was to evaluate the usefulness of two commercial products, ListShield™ and Listex™ P100, as biopreservatives to inhibit growth of *L. monocytogenes* in this traditional dry-cured ham. We also tested the ability of these products to reduce biofilms formed by this bacterium on two different types of surfaces, polystyrene and stainless steel, which are commonly used in the food industry. Our results show that phage preparations against *L. monocytogenes* can be successfully applied by the food industry for surface treatment and for reducing contamination of dry-cured ham.

2. Material and Methods

2.1. Bacterial strains, culture conditions and preparation of phage-based products.

Eleven *L. monocytogenes* strains from different origins were used in this study (Table 1) (Ortiz, López, & Martínez-Suárez, 2014). Bacteria were routinely cultured at 32ºC in TSB broth (Tryptic Soy Broth, Scharlau, Barcelona, Spain) or on TSB plates containing 2% (w/v) bacteriological agar (TSA). Selective growth of *Listeria* strains was performed on Oxford agar plates supplemented with 'Oxford-Listeria-selective supplement' (Merck Millipore, Darmstadt, Germany).

The working solutions of both phage products were prepared according to the suppliers' recommendations at the beginning of this study in 2014. Thus, ListShield™ (Intralytix, Inc., Baltimore, USA) was prepared by diluting the concentrated product 30 times in clean, sterile, chlorine-free water, whereas Listex™ P100 (Micreos, Wageningen,

Netherlands) was used without any further processing. From 2015 onwards, Micreos recommends a 0.2-1% solution of Listex™ P100 for applications on RTE meats (Micreos Food Safety, 2015).

2.2. Determination of the efficiency of plating (EOP) and minimal inhibitory concentration.

The host range of the two products, ListShield™ and Listex™ P100, was tested by determining their efficiency of plating (EOP) against the *L. monocytogenes* collection. A 100 µl volume of stationary-phase host culture (10^9 CFU/ml) was mixed with several dilutions of Listex™ P100 or ListShield™ in 3 ml of molten TSB top agar (0.7% agar). The resulting mixture was poured onto TSA plates, which were then incubated for 24 h at 32ºC. The EOP was calculated as the titer of the phage product on the tested strain compared to the titer on the reference strain *L. monocytogenes* S2, which showed the highest phage titers for both phage preparations. All experiments were performed in triplicate.

Additionally, the minimal inhibitory concentration (MIC) of ListShield™ and Listex™ P100 was determined in triplicate by the conventional broth microdilution technique. Briefly, serial two-fold dilutions of each phage-based product were made in TSB using 96-Well Microtiter™ Microplates (Thermo Scientific, Madrid, Spain). Each well was inoculated with 10^6 CFU of *L. monocytogenes*. The MIC was defined as the lowest concentration that inhibited visible bacterial growth after 24 h of incubation at 32°C.

2.3. Biofilm formation and biomass staining

Biofilm formation assays were carried out as described previously for other bacteria (Gutiérrez, Ruas-Madiedo, Martínez, Rodríguez, & García, 2014). Briefly, overnight cultures were diluted down to 10^7 CFU/ml in fresh TSB medium and 200 µl of this suspension (~10^6 CFU/well) were poured into each well of a 96-well polystyrene TC

Microwell 96U w/lid nunclon DSI plate (NUNC, Thermo Scientific, Madrid, Spain). Alternatively, biofilms were grown over 10 × 10 × 1 mm stainless steel coupons that were previously autoclaved, placed into a 24-well flat-bottom microtiter plate (NUNC, Thermo Scientific, Madrid, Spain) and inoculated with 1 ml (~10^6 CFU/well) of *Listeria* cultures. Biofilms were grown for 24 h and 10 days at 32ºC or 12ºC, respectively. Following the removal of the planktonic phase, wells were washed twice by adding 200 µl (96-well plates) or 1 ml (24-well plates) of sterile PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4; pH 7.4). 96-wells plates and coupons transferred to a new 24-well plate were air dried for 15 min.

Biomass was determined by a conventional staining technique with some modifications (Stepanovic, Cirkovic, Ranin, & Svabic-Vlahovic, 2004). The biofilms were stained for 15 min with 200 µl (96-well plates) or 1 ml (24-well plates) of crystal violet (0.1% w/v). The stain was further removed and the biofilms washed carefully with tap water before detaining with 200 µl (96-well plates) or 1 ml (24-well plates) of acetic acid (33% v/v) for 30 min. Absorbance was measured at 595 nm in a Microplate Benchmark Plus (BioRad, Hercules, CA) spectrophotometer. In the case of coupons, these were removed from the wells before the measurement.

2.4. ListShield™ and Listex™ P100 application against *Listeria* biofilms.

Biofilms grown on stainless steel or polystyrene were treated with either ListShield™ (10^7 PFU/well) or Listex™ P100 (10^9 PFU/well) for 4 h at 12ºC or 32ºC. Control biofilms were treated with SM buffer (20 mg/l Tris HCl, 10 mg/l $MgSO_4$, 10 mg/l $CaCl_2$ and 100 mg/l NaCl, pH 7.5). After treatment, wells were washed twice with PBS buffer and air-dried as described in section 2.3. Biomass was calculated by crystal violet staining (section 2.3) and the cell counts of the adhered cells were also determined as described previously (Gutiérrez, et al., 2015). Briefly, after washing the biofilms with

PBS and further air-drying (section 2.3), wells or coupons were scratched twice with two sterile swabs. Swabs were then suspended in 9 ml of SM buffer and vigorously shaken for 1 min. Serial dilutions were plated onto TSA solid medium for bacterial counting. Plates were incubated for 24 h at 32ºC.

2.5. *Listeria* contamination and phage application in Spanish dry-cured ham.

To prepare the inoculum for contaminating dry-cured ham samples, three overnight cultures of *L. monocytogenes* S2 were diluted 1:5 in 5 ml of fresh TSB medium and incubated at 32°C until an OD_{600} of approximately 0.4 (10^8 CFU/ml) was reached. Cultures were then centrifuged for 10 min at 10,000 rpm and the pellets resuspended in 5 ml of PBS buffer. Serial dilutions of the *L. monocytogenes* cell suspensions in PBS were then performed to inoculate the dry-cured ham slices. These slices had been previously cut into 1×1 cm, 1 mm thick uniform squares (0.1079 g) using a sterilized stainless steel cutter. The dry-cured ham squares were placed individually into 24 flat-bottom microtiter plates (Thermo Scientific, Madrid, Spain) and inoculated with 100 µl of serial dilutions of *L. monocytogenes* cells in PBS in order to provide an inoculation level of 10^5 CFU/cm^2, 10^4 CFU/cm^2 and 10^3 CFU/cm^2. These values correspond to contamination levels of 2.32×10^7 CFU, 2.32×10^6 CFU and 2.32×10^5 CFU per 25 g of dry-cured ham, respectively. Bacterial cells were distributed onto the ham slice surface and allowed to dry for 15 min. As a control of non-contaminated samples, three slices of dry-cured ham were inoculated with 200 µl of PBS buffer. Subsequently, ham slices were treated with 100 µl of either ListShield™ (10^7 PFU/cm^2) or Listex™ P100 (10^9 PFU/cm^2); the product was spread onto the slice and allowed to dry for 15 min. As a control of non-treated samples, 100 µl of PBS buffer were spread onto three ham slices previously contaminated with *L. monocytogenes* S2. Samples were then incubated for 14 days at 4ºC or 12ºC. Cell counts were performed on days 1, 3, 6, 8, 10 and 14

after treatment. Ham slices were aseptically transferred into stomacher bags fitted with filters (BagPage, BagSystem, Interscience, St-Nom-la-Breteche, France) containing 10 ml of PBS buffer and homogenized for 2 min in a stomacher (model 80, Seward Medical, London, UK). After homogenization, the solution was centrifuged for 10 min at 10,000 rpm and the pellet was resuspended in 1 ml of PBS buffer. Serial dilutions were carried out and 100 µl were spread onto Oxford agar plates. Plates were incubated for 48 h at 32ºC.

2.6. Statistical analysis.

A one-way analysis of variance (ANOVA) and the LSD test were performed to establish any significant differences regarding viable cell numbers and biomass between the control and the treated biofilms, and between the control and phage-treated dry-cured ham slices contaminated with *L. monocytogenes* strains. This analysis was also used to determine any difference between ListShield™ and Listex™ P100 treatments. Differences were expressed as means ± standard deviations and the level of significance was established at $P<0.05$ (SPSS 11.0 Software for Windows; Chicago, IL, USA).

3. Results

3.1. *In vitro* sensitivity of different *L. monocytogenes* strains to phage-based products.

The potential efficacy of two commercial phage-based products, ListShield™ and Listex™ P100, to inhibit the growth of eleven *L. monocytogenes* strains isolated from food (cheese, meat and fish) was evaluated by determining the efficiency of plating (EOP) and the minimum inhibitory concentration (MIC). EOP values indicated that all strains were sensitive to ListShield™. By contrast, four of the tested strains (*L. monocytogenes* Lm1, Lm2, Lm3 and Lm41) were not sensitive to phage P100 since no

isolated lysis plaques could be observed; therefore, EOP values could not be calculated for these strains (Table 1). Moreover, the EOP and MIC values revealed that the tested strains exhibited different degrees of sensitivity to the phage products. In the case of Listex™ P100, the EOP values were similar for all the sensitive strains (EOP~10^{-1}), except for *L. monocytogenes* S12-1 which showed the lowest value ($2.9 \times 10^{-2} \pm 4.1 \times 10^{-3}$). For ListShield™, similar low EOP values were obtained against *L. monocytogenes* strains isolated from the dairy environment (EOP~10^{-3}), with the exception of *L. monocytogenes* Lm22 and Lm41 ($6.1 \times 10^{-1} \pm 4.1 \times 10^{-2}$ and $2.1 \times 10^{-1} \pm 3.5 \times 10^{-2}$, respectively). Among the strains of meat origin, *L. monocytogenes* S12-1 showed the lowest EOP value ($5.4 \times 10^{-2} \pm 5.1 \times 10^{-3}$). For both phage products, all the EOP results were in accordance with the MIC values since the highest MIC values corresponded to the strains with the lowest EOP. Of note, a wider range of MIC values (up to 2 log units) was observed among the strains tested for Listex™ P100. Thus, strains with an EOP $<10^{-1}$ showed a MIC of 10^6 PFU/ml while MICs between 10^3 and 10^5 PFU/ml were detected in strains with an EOP $>10^{-1}$. The lowest MIC (10^3 PFU/ml) was shown by the strain S2. For the non-sensitive strains (Lm1, Lm2, Lm3 and Lm41) MIC values could not be determined. Regarding ListShield™, the strains with EOP $<10^{-1}$ showed an MIC of 10^7 PFU/ml, while the MIC values obtained for those strains with the highest EOP values were in the range of 10^5-10^6 PFU/ml.

3.2. ListShield™ and Listex™ P100 are effective for biofilm removal.

Initially, we determined the ability of *L. monocytogenes* strains to form biofilms on both polystyrene and stainless steel surfaces. For this purpose, microtiter wells and stainless steel coupons were inoculated with 10^6 CFU/well and incubated for 10 days at 12°C. All strains were able to form biofilms on both surfaces, although biomass values on stainless steel were up to 3-fold higher than those obtained on polystyrene (Fig. 1). The

strongest biofilm-forming strains on stainless steel, namely S2, S7-2 and INIA2530, reached values of absorbance around 3.7 after 10 days. On polystyrene, A_{595} values of up to 0.5 were detected in all strains. Most biofilms grew exponentially until day 3 on both surfaces and then reached the stationary phase. Moreover, the ability of the strains to grow on polystyrene and stainless steel was also assayed at 32ºC. After 24 h of incubation, the biofilms grown on stainless steel and polystyrene, respectively, reached the stationary phase, and showed absorbance values up to 1.2 and 7.3-fold higher than those obtained at 12ºC (Fig. S1). Additionally, the presence of biofilms grown at 4°C was also evaluated in both surfaces but no adhered bacteria were recovered (data not shown).

Once established that all strains could form biofilms on both polystyrene and stainless steel surfaces, we tested the efficacy of the bacteriophage-based products against *L. monocytogenes* preformed biofilms following the standard procedure. Polystyrene surface and the optimal growth temperature for *L. monocytogenes* were used for this purpose. Forty-eight h biofilms grown on polystyrene were treated with ListShield™ (10^7 PFU/well) or Listex™ P100 (10^9 PFU/well) at 32ºC (Fig. 2). A significant reduction in biomass was observed for all treated biofilms. However, treatment with Listex™ P100 resulted in an even greater biomass reduction (54-98%) compared to that obtained with ListShield™ (12-48%) (Fig. 2A). Indeed, there were statistically significant differences between the effect of the two products on the biofilms formed by *L. monocytogenes* Lm37, S2, S12-1, S4-2, S7-2 and INIA 2530 ($P<0.05$). The effectiveness of the treatment was confirmed by counting the bacterial cells that remained adhered to the well after the treatment (Fig. 2B). Listex™ P100 achieved the highest reduction (4.5 - 6.9 log units) for strains isolated from the meat and fish industries. This reduction level led cell counts under the detection limit (< 10

CFU/well). ListShield™, on the other hand, achieved a reduction from 0.25 to 0.86 log units. Of note, two of the strains (Lm3 and Lm37) did not show any decrease in adhered biomass, although the number of adhered cells (0.35 and 0.42 log units, respectively) was significantly reduced (Fig. 2A and 2B).

In order to simulate the environmental conditions found in the food industry, biofilms were grown for 72 h at 12ºC on stainless steel coupons and then treated for 4 h with the two products at the same temperature (Fig. 3). A significant reduction of the total biomass ($P<0.05$) was achieved with both products (Fig. 3A). Treatment with ListShield™ resulted in significant biomass reductions (30-63%) ($P< 0.05$) except in the case of strains Lm22 and S2 ($P> 0.05$). Moreover, with the only exception of strain Lm22, the adhered cell counts confirmed the biomass results since a reduction of 0.31 - 1.6 log units was detected (Fig. 3B). The decrease in total biomass (22-74%) after treatment with Listex™ P100 was similar to that obtained with ListShield™ and even higher (strains Lm22, S2, S12-1 and S7-2) (Fig. 3A). Regarding viable cell counts, removal of adhered cells below the detection limit was obtained for INIA2530 and significant reductions (2.7 to 4.9 log units) ($P<0.05$) were observed for the other strains (Fig. 3B).

3.3. ListShield™ and Listex™ P100 inhibit *L. monocytogenes* growth in Spanish dry-cured ham.

To test the efficacy of phage-based products as biopreservatives, dry-cured ham samples were experimentally contaminated with 10^5, 10^4 and 10^3 CFU/cm^2 of *L. monocytogenes* S2 and treated with ListShield™ (10^7 PFU/cm^2) or Listex™ P100 (10^9 PFU/cm^2) (Fig. 4). Samples were stored at 4°C or 12°C and viable cell counts of *L. monocytogenes* were monitored for 14 days. The levels of *L. monocytogenes* in dry-cured ham samples stored at 4°C and treated with bacteriophages remained significantly lower ($P<0.05$)

316 than in the untreated control samples (Figure 4A). Notably, Listex™ P100 reduced the
317 viable counts below the detection limit (<10 CFU/cm^2) after one day of treatment at all
318 the assayed inoculum levels. In contrast, ListShield™ turned out to be less effective in
319 the most contaminated samples. Indeed, a reduction of about 3.5 log units was observed
320 after 14 days in samples contaminated with 10^5 and 10^4 CFU/cm^2 (Figure 4A).
321 Storage of samples at 12°C reduced the effectiveness of ListShield™ as no differences
322 in *L. monocytogenes* growth were observed at high inoculum levels (10^5 and 10^4
323 CFU/cm^2) after 14 days of incubation. In samples with lower contamination levels (10^3
324 CFU/cm^2), a reduction in viable counts below the detection limit was obtained after 8
325 days of treatment (Figure 4B). In the case of Listex™ P100, effectiveness was not
326 affected by the storage temperature, with the exception of samples inoculated with 10^4
327 CFU/cm^2 in which there was a delay in bacteria eradication (Fig. 4B).
328

329 **4. Discussion**

330 Biofilms formed by *L. monocytogenes* are one of the factors involved in its persistence
331 in food environments, as they are highly resistant to the disinfectants commonly used in
332 the food industry (Pan, Breidt, & Kathariou, 2006; Saa-Ibusquiza, Herrera, & Cabo,
333 2011). In this regard, *L. monocytogenes* strains isolated from three industrial settings
334 (meat, fish and dairy) were tested for their sensitivity to ListShield™ and Listex™
335 P100. We found a broader sensitivity to ListShield™ , which is a cocktail of six phages,
336 as all the assayed strains (100%) were inhibited by this product, whereas four strains of
337 dairy origin turned out to be resistant to Listex™ P100 (phage P100). These results are
338 not surprising since phage cocktails are expected to have a broader host range compared
339 to single phage preparations. These data further support the idea that phage cocktails
340 may be more suitable for phage biocontrol applications, particularly for such a

genetically heterogeneous species as *L. monocytogenes.* Overall, these results were in accordance with the MIC values for Listex™ P100, which were lower in strains of meat origin than in those isolated from cheese, but quite similar for ListShield™.

When testing the suitability of the strains for the treatment of adhered bacterial communities, we found that all the *L. monocytogenes* strains used in this work have a good ability to form biofilms on both polystyrene and stainless steel surfaces. Moreover, all strains were able to adhere to these surfaces both at 12ºC (the temperature of cutting plants) and at 32ºC (the optimal growth temperature for *L. monocytogenes*). This result supports their persistence in food industrial facilities. Interestingly, a previous work indicated that surface physicochemical properties like the hydrophobicity of stainless steel correlate with a higher adhesion ability (Chavant, Martinie, Meylheuc, Bellon-Fontaine, & Hebraud, 2002).

As ListShield™ and Listex™ P100 are recommended by their commercial suppliers to eliminate or significantly reduce *L. monocytogenes* on equipment surfaces, we treated preformed biofilms of this bacterium with these products at 12ºC and 32ºC. Both products yielded significant biomass reductions compared with the control samples at the two tested temperatures. Overall, treatment at 12°C, which is more relevant for food industrial settings, favors biofilm removal by ListShield™, which is also active against a broader spectrum of strains. Regarding reductions in viable cells, the lower effectivity of ListShield™ compared with Listex™ P100 could be related to the different phage concentration of the working solutions (10^8 PFU/ml for ListShield™ and 10^{10} PFU/ml for Listex™ P100), as determined on our reference strain *L. monocytogenes* S2. Furthermore, it is possible that *L. monocytogenes* S2 is not sensitive to all bacteriophages present in the ListShield™ cocktail. This would decrease the number of PFU/ml that can effectively infect the target cells. Although this phenomenon might

366 appear as a shortcoming of phage mixtures, it is vastly compensated by their increased
367 host range.
368 Previous studies had shown similar results after treatment of *L. monocytogenes* biofilms
369 on stainless steel with phage P100 (3.5- to 5.4-log/cm^2 reduction) (Iacumin, et al., 2016;
370 Soni & Nannapaneni, 2010), as well as with phages LiMN4L, LiMN4p and LiMN17
371 against 7-day biofilms, which reduced adhered bacterial cells by 3-4.5 log units
372 (Ganegama-Arachchi, et al., 2013). Furthermore, analysis by epifluorescence
373 microscopy revealed that some viable cells remained following biofilm treatment with
374 phage P100 (Montañez-Izquierdo, Salas-Vázquez, & Rodrígez-Jerez, 2012). This
375 clearly indicates that other sanitization methodologies should be used in combination
376 with phages to achieve the desired level of disinfection. Moreover, the reduction of *L.*
377 *monocytogenes* biomass might be useful to enhance the efficacy of disinfectants.
378 Likewise, Listex^{TM} P100 showed higher antimicrobial effect compared with chemical
379 disinfectants when used on stainless steel surfaces simulating shallow grooves
380 (Chaitiemwong, Hazeleger, & Beumer, 2014).
381 In the present study, we also assessed the effectiveness of ListShield^{TM} and Listex^{TM}
382 P100 as biopreservatives for the traditional Spanish dry-cured ham. Both products were
383 evaluated for their ability to reduce *L. monocytogenes* levels on sliced dry-cured ham
384 during storage at 12ºC (simulating drying rooms) and 4ºC (simulating cold storage
385 rooms). In samples treated with ListShield^{TM} and stored at 4°C, there was an initial
386 reduction in the number of bacterial cells followed by re-growth. This phenomenon is
387 quite common in the treatment of foods with bacteriophages, although no phage-
388 resistant bacteria are generally isolated after treatment (Chibeu, et al., 2013; Guenther &
389 Loessner, 2011; Rodríguez-Rubio et al., 2015). Diffusion of phages in the food matrix,
390 especially in solid foods, is very limited. This might hinder the contact between phage

391 and target bacteria or even provoke the inactivation of phage particles, which results in
392 a different efficacy of phages as biopreservatives depending on the chemical
393 composition, pH and structure of the food matrix (Guenther & Loessner, 2011; Oliveira,
394 et al., 2014). The efficacy of ListShield™ treatment appears to be lower at higher
395 temperatures and levels of bacterial contamination. Thus, application of the phage
396 cocktail at 12ºC hardly exerted any control over the growth of the pathogen. For some
397 *Listeria* phages (P70-like and P100-like) a reduction in the plating and adsorption
398 efficiencies was observed after increasing the incubation temperature from 30°C to
399 37°C (Tokman et al., 2016). Moreover, the higher initial bacterial inocula (10^5 CFU/cm^2
400 and 10^4 CFU/cm^2), i. e., lower phage/bacteria ratio, seem to reduce the efficacy of
401 phages to remove *L. monocytogenes* in foods (Silva, Figueiredo, Miranda, & de Castro
402 Almeida, 2014; Iacumin, et al., 2016). Consequently, phage concentration should not be
403 less than 10^8 PFU/g or /cm^2, especially on solid foods (Guenther & Loessner, 2011). In
404 this regard, the lower effectiveness observed in the ListShield™ treatment might be due
405 to the lower phage/bacteria ratio used compared to that of the Listex™ P100 treatment.
406 However, as mentioned above, it cannot be excluded the importance of the different
407 nature of the two products (single phage versus phage cocktail). Our results using
408 Listex™ P100 as a biopreservative were similar, although no re-growing bacteria were
409 observed during the storage period at any of the assayed temperatures. Application of
410 Listex™ P100 significantly reduced viable bacteria to undetectable levels compared
411 with untreated controls at both 4°C and 12°C. There were significant reductions in *L.*
412 *monocytogenes* counts within the first 24 h of phage contact time. Indeed, application of
413 Listex™ P100 maintains *L. monocytogenes* below the detection limit (<10 CFU/cm^2)
414 throughout the 14-day storage period at temperatures under 12°C. At a storage
415 temperature of 4°C, the effectivity of ListShield™ is also valuable despite the

considerably lower phage concentration used. It is noteworthy that at low contamination rates of dry-cured ham slices (10^3 CFU/cm^2), that can occur during cutting or further manipulation, both products can lower bacterial levels below the detection limit (<10 CFU/ml). It has been estimated that *Listeria* contamination of ready-to eat food products is generally in the range of 100 CFU/g (Lianou & Sofos, 2007), which is fairly low. In that range, it would be expected that anti-listerial phage-based products would be very effective even without using very high phage concentrations. Although the effect of phages on the organoleptic properties of dry-cured ham should be evaluated, the efficacy of both phage-based products represents a promising strategy to enhance traditional disinfection processes for the control of pathogen contamination.

5. Conclusion

To our knowledge, this is the first study comparing the effectiveness of two commercial bacteriophage-based products in biopreservation of Spanish dry-cured ham. ListShield™ exhibited a better target range compared to Listex™ P100. On the other hand, Listex™ P100 appeared to be slightly more effective than ListShield™ in some applications (e.g., dry-cured ham treatment). However, it must be noted that (i) the challenge strain used in our experiments happened to be more susceptible to Listex™ P100 than it was to ListShield™, and (ii) we used a considerably higher phage concentration of Listex™ P100 compared to ListShield™. However, and despite the observed differences, our results indicate that both commercial products could be used successfully for application in food safety.

6. Acknowledgments

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8. Tables legends

Table 1. Sensitivity of *L. monocytogenes* strains to two commercial phage-based products. EOP is represented by the means ± standard deviation of three biological repeats. NA: Not applicable.

***Listeria monocytogenes* strain**	**Serotype**	**Origin**	**Efficiency of plating (EOP)**		**Minimal inhibitory concentration (MIC) (PFU/ml)**	
			Listex™ P100	ListShield™	Listex™ P100	ListShield™
Lm1	-	Cheese	0	$5.6\times10^{-3}\pm3.7\times10^{-4}$	NA	2.1×10^{7}
Lm2	-	Cheese	0	$5.4\times10^{-3}\pm6.7\times10^{-5}$	NA	2.1×10^{7}
Lm3	-	Cheese	0	$3.8\times10^{-3}\pm3.7\times10^{-4}$	NA	2.1×10^{7}
Lm22	-	Cheese	$4.8\times10^{-1}\pm8.9\times10^{-2}$	$6.1\times10^{-1}\pm4.1\times10^{-2}$	3.4×10^{5}	2.1×10^{5}
Lm37	-	Cheese	$1.9\times10^{-1}\pm3.4\times10^{-2}$	$4.3\times10^{-3}\pm 1.3\times10^{-4}$	3.4×10^{5}	2.1×10^{6}
Lm41	-	Cheese	0	$2.1\times10^{-1}\pm3.5\times10^{-2}$	NA	2.1×10^{5}
S2	1/2a	Meat	1	1	2.8×10^{3}	2.1×10^{5}
S4-2	1/2b	Meat	$6.6\times10^{-1}\pm3.4\times10^{-2}$	$6.1\times10^{-1}\pm2.1\times10^{-2}$	3.4×10^{5}	2.1×10^{6}
S7-2	4b	Meat	$4.7\times10^{-1}\pm4.1\times10^{-2}$	$2.3\times10^{-1}\pm1.1\times10^{-2}$	3.4×10^{5}	2.1×10^{6}
S12-1	1/2c	Meat	$2.9\times10^{-2}\pm4.1\times10^{-3}$	$5.4\times10^{-2}\pm 5.1\times10^{-3}$	3.7×10^{6}	2.1×10^{7}
INIA2530	1/2a	Fish	$5.2\times10^{-1}\pm4.1\times10^{-2}$	$3.9\times10^{-1}\pm2.4\times10^{-2}$	3.1×10^{4}	2.1×10^{5}

9. Figures legends

Figure 1. Growth curves of biofilms formed by *L. monocytogenes* strains on polystyrene (■) and stainless steel surfaces (●) at 12°C. Total biomass was determined by crystal violet staining and subsequent measurement of absorbance at a wavelength of 595 nm. Values represent mean ± standard deviation of three biological replicates.

Figure 2. Removal of 48-old biofilms formed on polystyrene by treatment with ListShield™ (grey) and Listex™ P100 (white) for 4 h at 32°C. A) Biomass removal expressed as percentage of absorbance reduction after treatment with phage-based products. B) Viable cell counts are expressed as log CFU/well. Non-treated biofilms were used as controls (black bars). Bars having an asterisk (*) show a statistically significant difference from the control (P<0.05). Bars marked with a letter "a" represent statistically significant differences between treatments (P<0.05). Values represent the means ± standard deviations of three biological replicates. Listex™ P100 was not tested against biofilms formed by strains *L. monocytogenes* Lm1, Lm2, Lm3 and Lm41 since they are not sensitive to phage P100.

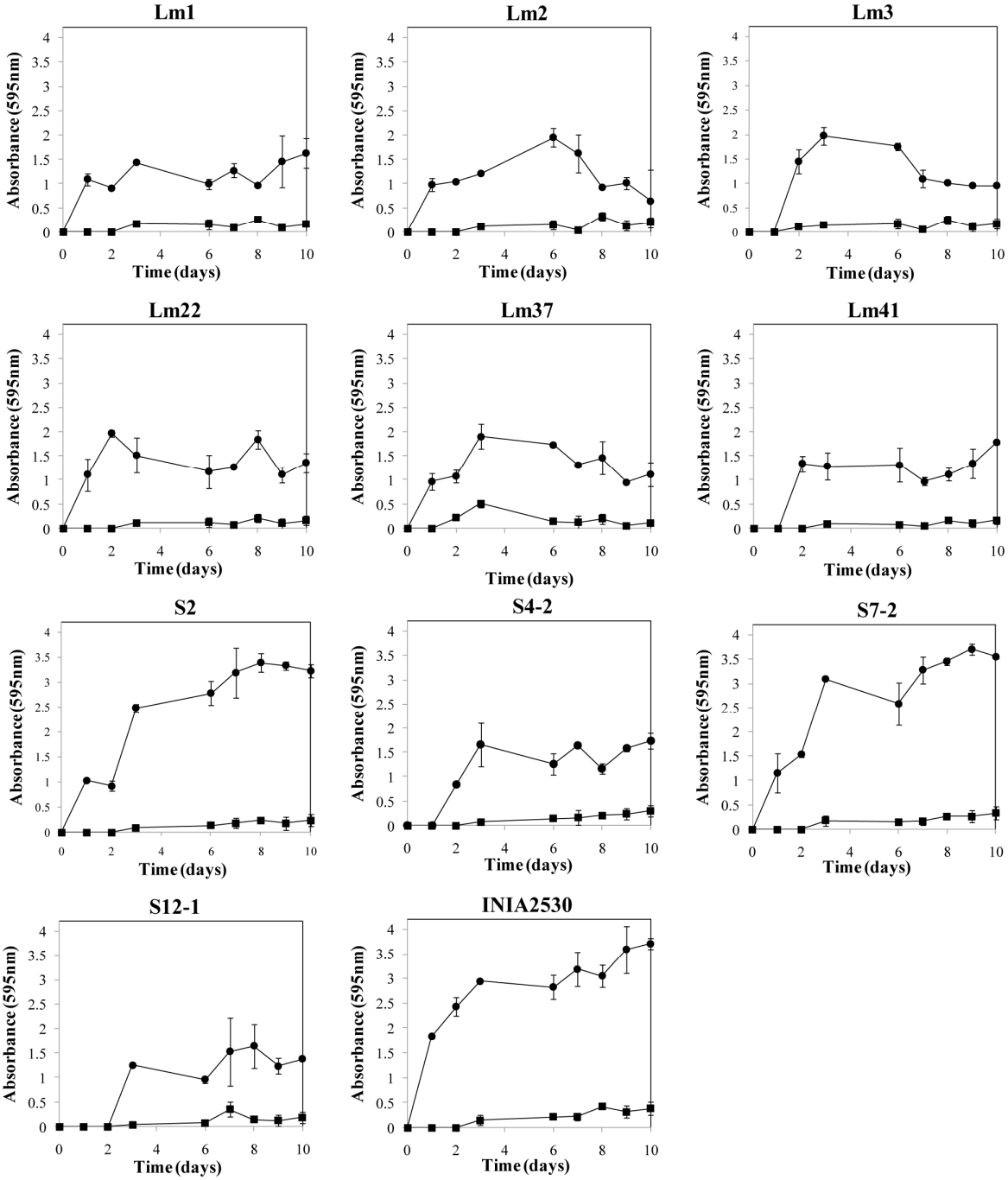
Figure 3. Removal of 72 h-old biofilms formed on stainless steel by treatment with ListShield™ (grey) and Listex™ P100 (white) at 12°C for 4 h. A) Biomass removal expressed as percentage of absorbance reduction after phage-based products treatment. B) Viable cell counts are expressed as log CFU/well. Non-treated biofilms were used as controls (black bars). Bars having an asterisk (*) represent statistically significant differences from the control (P<0.05). Bars marked with a letter "a" show statistically significant differences between treatments (P<0.05). Values are means ± standard deviations of three biological replicates. Listex™ P100 was not tested against biofilms

formed by *L. monocytogenes* strains Lm1, Lm2, Lm3 and Lm41 since they are not sensitive to phage P100.

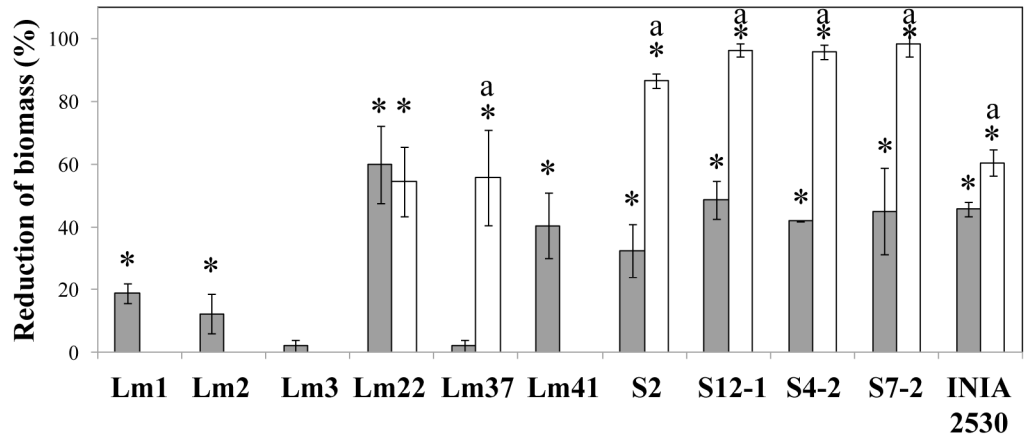
Figure 4. Effect of phage-based products on *L. monocytogenes* levels in Spanish dry-cured ham stored at 4°C (A) and 12ºC (B). Samples were deliberately contaminated with 10^5 CFU/cm^2 (I), 10^4 CFU/cm^2 (II) and 10^3 CFU/cm^2 (III) of *L. monocytogenes* S2. Control (●); treated with Listex^{TM} P100, 10^9 PFU/cm^2 (▲); treated with ListShield^{TM}, 10^7 PFU/cm^2 (■). Values represent means ± standard deviations of three biological replicates. Bacteria detection threshold is 10 CFU/cm^2.

10. Supplementary material.

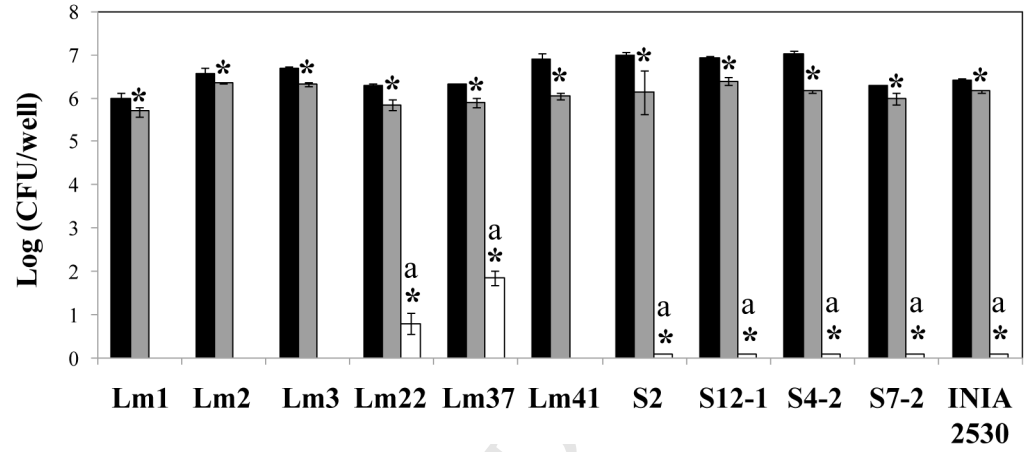
Figure S1. Growth curves of biofilms formed by *L. monocytogenes* strains on polystyrene (■) and stainless steel surfaces (●) at 32°C. Total biomass was determined by crystal violet staining and subsequent measurement of absorbance at a wavelength of 595 nm. Values represent mean ± standard deviation of three biological replicates.


Lm1
Lm2
Lm3
Lm22
Lm37
Lm41
S2
S4-2
S7-2
S12-1
INIA2530
Absorbance (595nm)
Time (days)

A



B



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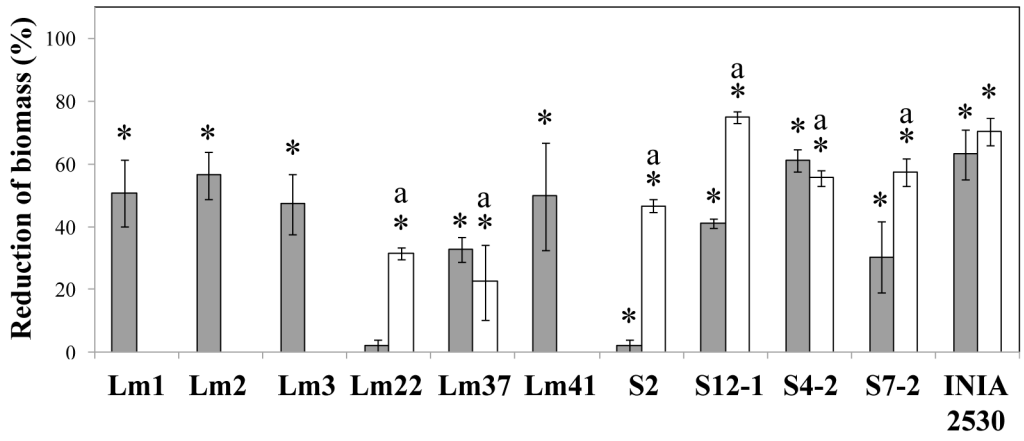

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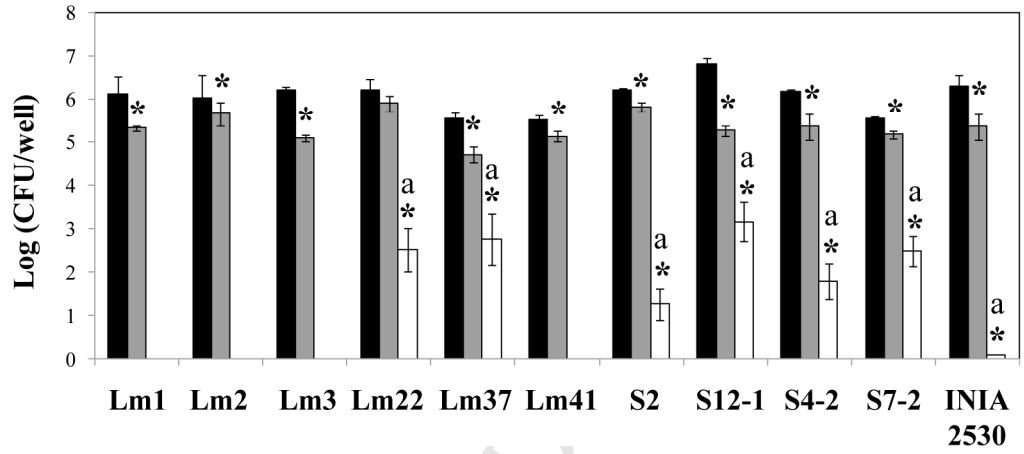

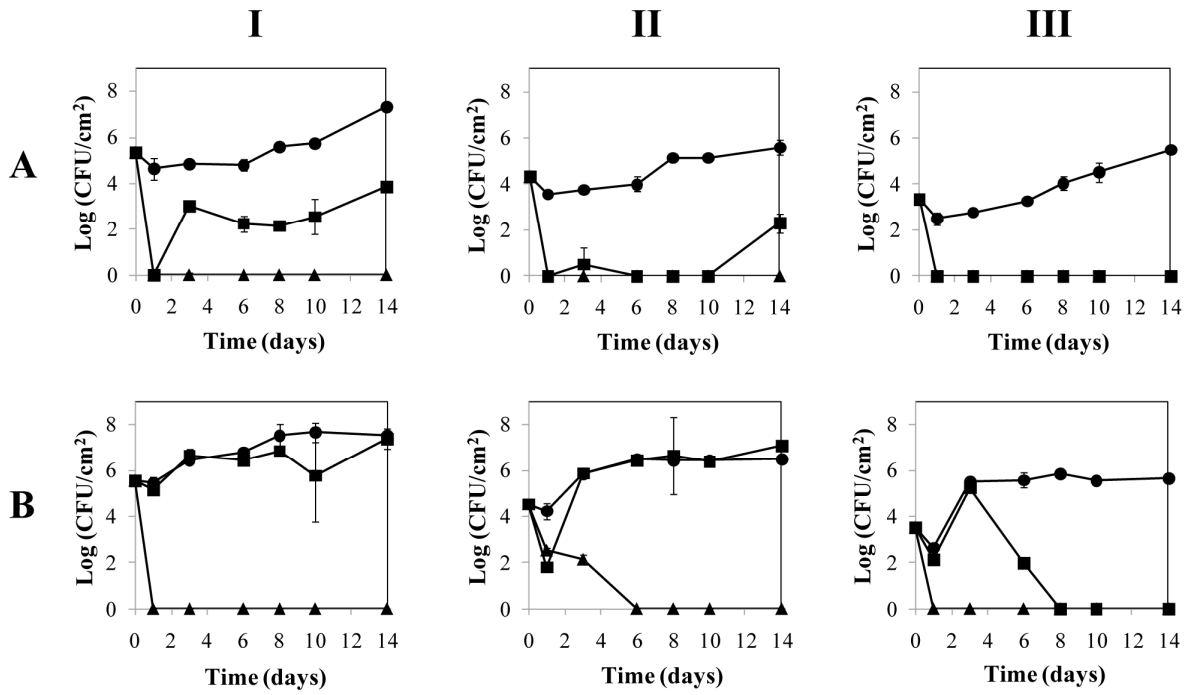

Highlights

- ListShield™ and Listex™ P100 were effective to improve food safety.
- Phage disinfection success is dependent on the phage titer of each product.
- Effectiveness of phage-based products is reliant on the target strains sensitivity.
- Commercial phage-based products could prevent low contamination of dry-cured ham.